

# Efficient retrovirus-mediated transfer of the multidrug resistance 1 gene into autologous human long-term repopulating hematopoietic stem cells

RAFAT ABONOUR<sup>1</sup>, DAVID A. WILLIAMS<sup>2,3,4</sup>, LAWRENCE EINHORN<sup>1</sup>, KRISTIN M. HALL<sup>5</sup>, JUN CHEN<sup>5</sup>, JOHN COFFMAN<sup>5</sup>, CHRISTIE M. TRAYCOFF<sup>1</sup>, ARTHUR BANK<sup>5</sup>, IKUNOSHIN KATO<sup>6</sup>, MAUREEN WARD<sup>5</sup>, STEPHEN D. WILLIAMS<sup>1</sup>, ROBERT HROMAS<sup>1,2,7</sup>, MICHAEL J. ROBERTSON<sup>1</sup>, FRANKLIN O. SMITH<sup>1,2,3</sup>, DAVID WOO<sup>8</sup>, BONNIE MILLS<sup>9</sup>, EDWARD F. SROUR<sup>1,2</sup> & KENNETH CORNETTA<sup>1,2,7</sup>

<sup>1</sup>Department of Medicine, <sup>2</sup>Department of Microbiology and Immunology, <sup>3</sup>Department of Pediatrics and

<sup>4</sup>Herman B Wells Center for Pediatrics Research, Howard Hughes Medical Institute,

Indiana University School of Medicine, 1044 W. Walnut, Rm. 402, Indianapolis, IN, 46202, USA

<sup>5</sup>Columbia University, 701<sup>st</sup> West 168<sup>th</sup> St., HHSC16-1604, New York, New York, 10032, USA

<sup>6</sup>Takara Shuzo, Biomedical Group, Seta 3-4-1, Otsu, Shiga, Japan 520-2193

<sup>7</sup>Department of Medical and Molecular Genetics, Indiana University School of Medicine, 1044 W. Walnut, Rm. 202, Indianapolis, Indiana 46202, USA

<sup>8</sup>AMGEN, One Amgen Center Drive, Thousand Oaks, California 91320-1799, USA

<sup>9</sup>Nexell Therapeutics, 9 Parker, Irvine, California 92618, USA

**Pre-clinical studies indicate that efficient retrovirus-mediated gene transfer into hematopoietic stem cells and progenitor cells can be achieved by co-localizing retroviral particles and target cells on specific adhesion domains of fibronectin. In this pilot study, we used this technique to transfer the human multidrug resistance 1 gene into stem and progenitor cells of patients with germ cell tumors undergoing autologous transplantation. There was efficient gene transfer into stem and progenitor cells in the presence of recombinant fibronectin fragment CH-296. The infusion of these cells was associated with no harmful effects and led to prompt hematopoietic recovery. There was *in vivo* vector expression, but it may have been limited by the high rate of aberrant splicing of the multidrug resistance 1 gene in the vector. Gene marking has persisted more than a year at levels higher than previously reported in humans.**

The successful transfer and expression of new genetic sequences in hematopoietic stem and progenitor cells may improve the management of both malignant and non-malignant human conditions<sup>1-3</sup>. Retrovirus-mediated gene transfer is widely used for this purpose because it has the potential for stable vector integration and expression<sup>4</sup>. Unfortunately, retrovirus-mediated gene transfer into adult human stem and progenitor cells has proven problematic, and clinical application of this therapy has been limited by low gene transfer into long-term repopulating cells<sup>5-8</sup>. Several modifications have recently led to improved gene transfer into stem cells, including the use of newly discovered early acting cytokines, highly expressed alternative viral receptors, modified vectors, 'activated' bone marrow cells obtained after mobilization induced by growth factor, and co-localization of stem cells and vector particles by centrifugation or by transduction in the presence of autologous stroma or fibronectin<sup>9-18</sup>.

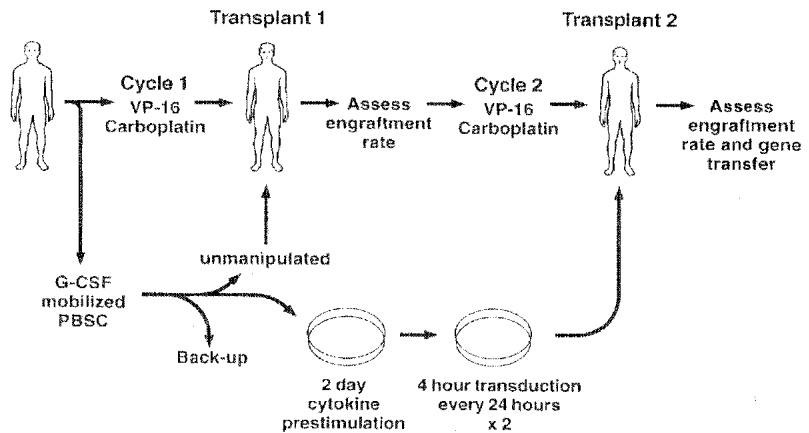
We based our protocol on recent observations that fibronectin-mediated co-localization of target cells and retroviral particles increases gene transfer efficiency. Fibronectin contains specific adhesion domains for stem and progenitor cells and retroviral vectors and can increase the apparent titer of retroviral particles presented to target cells. CH-296, a chimeric molecule that contains these domains, has been associated

with the highest efficiency of gene transduction<sup>19</sup>. Studies in nonhuman primates and a non-obese diabetic/severe combined immunodeficiency mouse model of human disease have demonstrated better gene transfer with CH-296 than with co-cultivation or vector supernatant infection alone<sup>15,20</sup>.

For our trial, we enrolled patients undergoing autologous transplantation for germ cell tumors. These tumors have become a model for curable malignant disease, with more than 70% of patients with advanced disease cured by first line therapy. Patients with relapsed or refractory disease have a lower rate of being cured. The addition of high-dose carboplatin and etoposide plus rescue with autologous peripheral blood progenitor cells (PBPCs) successfully cures a substantial portion of relapsed patients. Moreover, the addition of three cycles of oral etoposide after transplantation further improves the outcome of patients with relapsed germ cell tumors<sup>21</sup>. However, myelosuppression has been a principal limitation, resulting in dose modification and treatment delay. The expression of the gene for human multidrug resistance 1 (MDR-1) in hematopoietic progenitor cells may render these cells resistant to oral etoposide<sup>22,23</sup>, thus allowing for 'dose-intensive' therapy without delay, with the intention of further improving the survival rate of patients with germ cell tumors.

To begin to address this, we initiated a phase I study. To reduce

**Fig. 1** Treatment protocol. Peripheral blood progenitor cells were mobilized using G-CSF. These cells were divided into three groups. The first was not manipulated and was infused after the first cycle of high-dose chemotherapy. The second was used for gene manipulation and was the sole source for hematopoietic reconstitution after the second cycle of high-dose chemotherapy. After CD34<sup>+</sup> cell selection, cells were maintained in cytokine-containing media. Transduction was done on days 3 and 4 with clinical grade vector using CH-296. After etoposide (VP-16) transplantation, patients received three cycles of oral (not shown).



the risk that the MDR-1 might be inadvertently introduced into malignant cells<sup>24</sup>, we first purified patients' peripheral blood stem and progenitor cells using the CD34 antigen, which is expressed on stem and progenitor cells but not on adult germ cell tumor cells<sup>25</sup>. Our study design allowed for the comparison of engraftment kinetics of unmanipulated and *ex vivo*-manipulated cells in each patient. In addition, the availability of a large database at our institution allowed comparison with a similar patient population that did not receive manipulated cells.

Here, autologous peripheral blood CD34<sup>+</sup> cells transduced with MDR-1 in the presence of CH-296 engrafted in 12 patients undergoing autologous transplantation for germ cell tumor. There was not consistent MDR-1 expression, related in part to the presence of splicing signals in the MDR-1 vector, which may result in the generation of truncated, non-functional P glycoprotein<sup>26,27</sup>. However, the frequency of gene transfer, as measured by the presence of proviral-containing progenitors in patients' bone marrow and circulating white cells, was higher than in previously reported human trials of MDR-1. Moreover, vector-transduced hematopoietic cells derived from primitive progenitor cells persisted at high levels in most patients for at least 1 year. The transduction and transplantation protocol is a substantial advance in the use of retroviral vector to transduce human hematopoietic progenitor cells.

#### Patients and mobilization characteristics

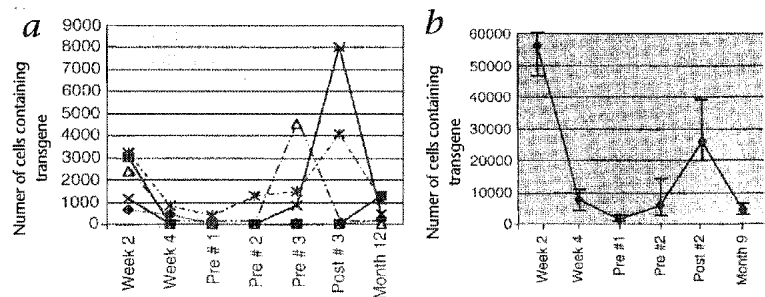
We enrolled twelve patients eligible for tandem transplantation (median age, 29 years (range, 17–51); Fig. 1, treatment protocol). PBPCs were mobilized using 10 µg granulocyte colony-stimulating factor (G-CSF)/kg per day, and were divided into three groups. The first was unmanipulated and used for the initial transplant. The second was selected by CD34<sup>+</sup>, stimulated with cytokines, transduced with MDR-1 retroviral vector and then cryopreserved; this served as the only source of cells for

the second transplant. The third, unmanipulated group was cryopreserved for use in case of graft failure. Mobilization was successful in 11 patients. For one patient, an insufficient number of cells was mobilized to meet the minimal requirements for this protocol, and the patient successfully completed tandem transplantation using unmanipulated PBPCs. One leukapheresis product from this patient was transduced; gene transfer data is included in this analysis.

#### CD34<sup>+</sup> cell transduction

To facilitate transduction, CD34<sup>+</sup> cells were maintained *ex vivo* in the presence of cytokines for 5 days. Cells were cultured with stem cell factor and interleukin 6 (SCF/IL-6) in the first ten patients, and with stem cell factor, megakaryocyte growth and differentiation factor and G-CSF (SCF/MGDF/G-CSF) in the final two patients. On days 3 and 4 of *ex vivo* stimulation, cells were cultured with MDR-1 vector on plates coated with CH-296 for 4 hours each day. After vector exposure, the cells were supplied with fresh media and cytokines and were incubated overnight on dishes coated with CH-296. Manipulated cells were cryopreserved on day 5. When CD34<sup>+</sup> cells were cultured in the presence of SCF/IL-6 (10 patients requiring 17 separate transduction procedures), there was no consistent increase in cell number. In eleven procedures there was a loss in CD34<sup>+</sup> cells of 4–62%, whereas there was a gain in CD34<sup>+</sup> cells of 3–52% in six transductions. Changes in total cell numbers paralleled changes in CD34<sup>+</sup> cell numbers using SCF/IL-6. In three separate transductions for the two patients in whom SCF/MGDF/G-CSF was used, there was an increase in total nucleated cells of 88% (range, 80–99%) and an increase in CD34<sup>+</sup> cells of 39% (range, 30–42%).

**Fig. 2** Transgene frequency in peripheral blood leukocytes as determined by quantitative real-time PCR. Values (as transgene-containing cells/10<sup>6</sup> cells) represent the median of three independent analyses. **a**, Peripheral blood leukocytes from patients whose CD34<sup>+</sup> cells were transduced in the presence of SCF/IL-6. ♦, patient 5; ■, patient 6; ▲, patient 8; ×, patient 9; \*, patient 10. **b**, Peripheral blood leukocytes from patient 12, whose CD34<sup>+</sup> cells were transduced in the presence of SCF/MGDF/G-CSF. Pre #1, Pre #2 and Pre #3, samples obtained before first, second and third cycles of oral etoposide, respectively; Post #3, sample obtained 6–8 weeks after the last cycle of oral etoposide. Scales for vertical axes in **a** and **b** differ.



## ARTICLES

**Table 1** Hematopoietic recovery

	Transplant 1 (Unmanipulated cells)	Transplant 2 (Transduced cells only)	Transplant 2 (“Historical” control)
CD34 <sup>+</sup> cell dose/kg	3.5 × 10 <sup>6</sup> (1–9.7)	3.2 × 10 <sup>6</sup> (2.3–5.7)	3.5 × 10 <sup>6</sup> (1–14)
Days to ANC > 0.5 × 10 <sup>9</sup>	10 (8–11)	9 (8–11)	11 (8–13)
Days to platelets > 20 × 10 <sup>9</sup>	10 (10–14)	13 (10–35)	13 (8–36)

Both unmanipulated and transduced products were infused without toxicity. Data include the number of cells infused per kg body weight, and the number of days until the absolute neutrophil count (ANC) or platelet count reached more than 0.5 × 10<sup>9</sup> cells/l or 20 × 10<sup>9</sup> platelets/l, respectively (ranges, in parentheses). “Historical” control, 25 consecutive patients with germ cell tumors treated in an identical way without receiving manipulated cells.

**Assessment of early and late hematopoietic function**

Eleven patients completed the tandem transplantation regimen; we used unmanipulated PBPCs during the first transplant, and MDR-1-transduced CD34<sup>+</sup> cells for the second transplant. The median numbers of CD34<sup>+</sup> cells/kg were 3.5 × 10<sup>6</sup> and 3.2 × 10<sup>6</sup> for the first and second transplants, respectively (Table 1). Both unmanipulated and transduced products were administered without infusion-related toxicity. Hematopoietic recovery rates after the first and second transplants were similar. The times to absolute neutrophil count above 0.5 × 10<sup>9</sup> cells/l were 10 days for the first transplant (unmanipulated cells) and 9 days for the second transplant (stimulated and vector-transduced cells). Platelet recovery was also similar for the first and second transplants. We compared the hematopoietic recoveries of these 11 patients with those of a control group of 25 consecutive patients with germ cell tumor treated in an identical way but not receiving manipulated cells. There were no differences in neutrophil or platelet recovery after CD34<sup>+</sup> cell selection, cytokine stimulation, exposure to CH-296 and vector transduction (Table 1).

Complete blood counts of eight patients on samples obtained more than 1 year after infusion of cells transduced with MDR-1 showed all patients had normal hemoglobin levels, white blood cell counts and differentials (data not shown). Platelet counts were within normal range except for one patient, who had a slight reduction in platelets, to 103 × 10<sup>9</sup> platelets/l (normal, 150 × 10<sup>9</sup>–450 × 10<sup>9</sup> platelets/l).

**Gene transduction efficiency**

We plated transduced cells in methylcellulose and counted progenitor colonies on day 14. We defined gene transfer efficiency as the percentage of progenitor colonies containing the MDR-1 vector, as assessed by PCR for individually isolated colonies. We screened a median of 52 colonies for each patient's transduced CD34<sup>+</sup> cells (range, 27–87), which included a median of 30 erythroid burst-forming units (range, 6–53) and a median of 19 granulocyte-monocyte colony-forming units (CFU) (range, 8–36 colonies). The median gene transfer efficiency of all colonies immediately after transduction was 14% (range, 4–52%), and was 24% for erythroid burst-forming units (4–47%) and 13% for granulocyte-monocyte CFU (0–67%) (Table 2). To confirm the preclinical data demonstrating the drug selection potential of the A12M1 vector<sup>28</sup>,

we plated cells from patient 10 in the presence or absence of the anti-tumor agent paclitaxel, at a concentration of 10 ng/ml. “Control-transduced” CD34<sup>+</sup> cells lacked the MDR-1 transgene, by PCR, and did not form colonies in the presence of paclitaxel. In contrast, 23% of transduced progenitors survived this exposure and all surviving colonies contained the transgene, by PCR analysis. Gene transduction efficiency (measured by PCR) in this patient was 52%, demonstrating that close to 50% of this patient's transduced colony-forming cells expressed functional MDR-1.

**Engraftment of transduced cells**

At 1 month after infusion, the median frequency of colonies containing the transgene in aspirated bone marrow was 12% (range, 0–78%). The percent of transduced progenitor cells in the bone marrow was maintained at 9% (range, 5–14%) and 8% (range, 3–10%) during the period of maintenance chemotherapy at 3 and 6 months after infusion, respectively. The percent of transgene-containing bone marrow progenitors was 0–15% in seven patients that could be evaluated at 12 months, with two patients demonstrating more than 10% colonies containing the transgene by PCR at that time (Table 3).

We used analyses of peripheral blood by nested PCR and quantitative real-time PCR to determine the presence of transgene in nucleated blood cells. Using nested PCR capable of detecting full-length and truncated MDR-1, we detected the presence of transgene in all 11 patients on day 14 after infusion of transduced cells. At 1 month after infusion, 8 of 11 patients had vector-containing cells in the peripheral blood. The results of nested PCR became negative in all patients in subsequent months; however, after the completion of maintenance chemotherapy, vector-containing cells reappeared in the circulation in four of nine patients. These four patients have maintained detectable levels (5–15%) of transgene-containing colonies in their bone marrow samples 1 year after infusion of cells.

To quantify the level of gene-transduced cells in the peripheral blood, we used real-time PCR with primers that detect the full-length MDR-1. We detected transgene-containing nucleated cells in seven patients up to 12 months after transplanta-

**Table 2** Gene transduction efficiency

Patient	%(+) colonies	Total <sup>a</sup>	% BFU-E <sup>b</sup>	Total <sup>b</sup>	%CFU-GM <sup>c</sup>	Total <sup>c</sup>
1	15	27	33	6	12	17
2	34	47	33	33	36	14
3	31	36	28	25	50	8
4	4	50	7	29	0	18
5	6	83	7	51	13	32
6	8	87	12	53	3	36
7	12	52	13	30	9	22
8	5	60	4	28	0	21
9	9	50	12	26	7	29
10	52	58	47	43	67	15
11	31	52	29	41	31	13
12	27	60	37	30	17	30
Median	14	52	24	30	13	19

<sup>a</sup>Total number of colonies (granulocyte-monocyte CFU; granulocyte, erythroid, macrophage and megakaryocyte CFU; and erythroid burst-forming units) screened per patient. <sup>b</sup>Percent positive colonies and total erythroid burst-forming units analyzed. <sup>c</sup>Percent positive colonies and total granulocyte-monocyte CFU analyzed. BFU-E, erythroid burst-forming units; CFU-GM, granulocyte-monocyte CFU.

**Table 3** Percentage of transgene-containing colonies in the graft and at 4 time points after transplantation

Subject	Graft	1 month	Pre cycle 2 <sup>a</sup>	Post cycle 3 <sup>b</sup>	1 year
1	15	ND	5	NA	NA
3	31	14	10	9	3
4	4	0	8	10	0
5	6	28	15	9	5
6	8	9	14	8	4
7	12	8	NA	NA	NA
8	5	14	7	4	15
9	9	8	NA	8	13
10	52	8	ND	3	7
11	31	78	NA	NA	NA
12	27	31	9	7	NA

<sup>a</sup>Timing for obtaining samples before cycle 2 of oral etoposide (VP16) (planned *in vivo* selection for transduced cells): 2 months after transplant of transduced cells in three patients, 3 months in three patients, and 5 months in 1 patient. <sup>b</sup>Timing for obtaining samples after cycle 3 of oral etoposide (VP16): 5 months in one patient, 4 months in two patients and 6 months in five patients. ND, not determined; NA, samples not available for analysis because of disease progression ( $n = 5$ ) or patient refusal ( $n = 3$ ) or because samples were not delivered ( $n = 2$ ).

tion (Fig. 2). For the two patients (11 and 12) whose CD34<sup>+</sup> cells were pre-stimulated and transduced using SCF/MGDF/G-CSF, we detected 5.61% and 4.30% transgene-containing nucleated cells in the blood during the first month after transplantation. Maintenance chemotherapy for these two patients was delayed because of intervening surgical procedures to remove residual tumor masses. Patient 12 received only two cycles, because of disease progression. In this patient, transgene-containing cells decreased from 5.61% to 0.13% before *in vivo* exposure to etoposide. After the first and second cycles of oral etoposide, circulating transgene-containing cells increased to 0.58% and 2.60%, respectively, indicating an increase of 450% with each cycle. Patient 11 was not compliant with oral etoposide and received only part of the first cycle. The circulating transgene-containing cells continued to decrease with time in the absence of exposure to etoposide, from 4.3% immediately after transplantation to 0.1% 8 months later (data not shown). Patients 8, 9 and 10 had low levels of transduced cells (range, 0.1–0.3%) in the peripheral blood shortly after transplantation (Fig. 2a). After receiving oral etoposide, they showed an augmentation in the number of circulating transduced cells (an increase of 300–900% in transgene-containing cells). These data indicate *in vivo* selection of transduced cells.

The MDR-1 vector used here contained both truncated and full-length transgenes because of splice sites in the MDR-1 cDNA. To determine transgene integrity, we analyzed transduced cells and samples obtained after transplant by PCR using two primers sets that allow for the detection of either full-length or truncated MDR-1 integrated DNA. We detected both truncated and full-length versions in the transduced products (Fig. 3). Unexpectedly, PCR analysis of peripheral blood samples on day 14 showed only the truncated transgene in 6 out of 11 patients. The ratio of full-length transgene to truncated transgene isoforms during the first month after transplantation in four separate analyses from patient 12 was 1:2–1:10.

To determine whether a functional P-glycoprotein was present at later times, we cultured bone marrow cells with or without paclitaxel. Cryopreserved samples from five patients at 1 year and a sample from one patient at 9 months (time of disease progression) were available for analysis. We detected no colonies in three patients and five control samples grown in the

presence of paclitaxel. In the other three patients (patients 8, 10 and 12), 4, 2 and 6 colonies per  $2 \times 10^5$  bone marrow cells, respectively, grew in the presence of paclitaxel, whereas we counted 32, 8 and 40 colonies per  $2 \times 10^5$  bone marrow cells, respectively, in the absence of paclitaxel. All colonies grown in the presence of paclitaxel contained vector DNA, as shown by PCR. These data indicate the presence of a functioning transgene in these patients 1 year after transplantation.

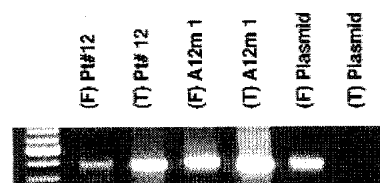
### Clinical outcome

The median follow-up at this analysis was 18 months from the infusion of transduced cells. Three of the eleven patients have died from progressive germ cell tumors. One patient relapsed and obtained a remission using further chemotherapy. Eight patients are alive with normal marrow cellularity and function when assessed at 1 year after transplantation. Of the 33 planned cycles of maintenance etoposide, 28 were given. Only one was withheld because of prolonged cytopenia. Four were withheld because of disease progression. In addition, one patient required early termination of cycle 3 because of neutropenic fever.

We assessed treatment-related toxicity by frequent clinical and laboratory evaluations. So far, there have been no unanticipated adverse clinical consequences. We detected neither viral envelope nor replication-competent retroviruses in transduced CD34<sup>+</sup> cells before infusion or in any samples obtained after transplantation. We have used both enzyme-linked immunosorbent assay (ELISA) and western blot analysis to assess the development of antibodies against CH-296. We obtained samples eight times during the first year after infusion of transduced cells. We also obtained samples before transplantation for baseline analysis. We re-assayed, using western blot analysis, five samples that exceeded the established ELISA titer limit. Of these, one sample from patient 3, obtained 1 year after transplantation, produced weakly positive results. The other eight samples from this patient produced negative results by ELISA and western blot analysis. No sample before transplantation was available from this patient, who has no clinical symptoms and is in good health 28 months after infusion of transduced cells.

### Discussion

Transplantation of cytokine-stimulated CD34<sup>+</sup> cells transduced with AM12 MDR-1 retroviral vector in the presence of recombinant fibronectin CH-296 was associated with prompt hematopoietic recovery and no adverse events. With more



**Fig. 3** Transgene integrity. Samples from transduced CD34<sup>+</sup> cells from patient 12 (Pt#12), producer cell line (A12m1) and plasmid containing MDR-1 cDNA (Plasmid) were analyzed by PCR for the presence of truncated (T) and full-length (F) MDR-1. As expected, the plasmid contains only full-length MDR-1, whereas A12m1 and the patient's sample contain both full-length and truncated transgene.

## ARTICLES

than 1 year of follow-up, we have demonstrated the continued contribution of vector-derived cells to hematopoiesis. The level of long-term engraftment of retrovirally transduced peripheral blood progenitor cells was the highest reported so far.

Here, the exposure of CD34<sup>+</sup> cells to the A12M1 vector with CH296 yielded efficient gene transfer. The A12M1 vector has been used before with less-impressive gene transfer efficiencies. In another study<sup>5</sup>, five patients were transplanted with a mixture of untransduced and CD34<sup>+</sup>-enriched stem cells transduced on plates coated with whole fibronectin (not CH-296). The latest time at which vector-transduced cells were detected by PCR was 10 weeks after transplantation<sup>5</sup>. There was only a transient and low rate of engraftment of MDR-1-transduced CD34<sup>+</sup> cells in 5 of 20 patients using either suspension or stroma methodology<sup>6</sup>. Stroma was also used to aid gene transfer in a study of six patients; half of the enriched CD34<sup>+</sup> cells were transduced with an MDR-1 vector and the other half with a marking vector (NeoR) (ref. 29). There was low-level gene marking of granulocytes (0.01–1%, by semi-quantitative PCR), with one of these six patients showing NeoR marking and two patients showing MDR-1-marked-granulocytes close to 6 months after transplantation<sup>29</sup>. In a second study, four patients received CD34<sup>+</sup> cells transduced with the MDR-1 vector along with unmanipulated cells<sup>30</sup>. One patient had detectable MDR-1 transgene in granulocytes (by semi-quantitative PCR) at almost 4 months; no long-term data was provided<sup>30</sup>. The ultimate goal of this gene therapy approach is decreased cytopenia after chemotherapy administration. Although the patients in our study seem to have tolerated after transplant chemotherapy better than patients in other studies have, the possibility of patient selection bias cannot be excluded and therefore the contribution of transduced cells to the clinical outcomes is unclear<sup>21</sup>.

Improved transduction efficiency in the presence of matrix proteins is well-described, and the advantages of CH-296-assisted gene transfer include ease of use and safety<sup>20,31–33</sup>. This approach does not pose the technical problems associated with autologous or allogeneic stroma<sup>34</sup>. Also, recent data indicate the preservation of long-term repopulating cells in *ex vivo* cultures that contain matrix protein such as fibronectin<sup>35</sup>. Transplantation models in nonhuman primates have noted improved transduction of long-lived hematopoietic progenitor cells with results similar to our findings in humans. In one study, the frequency *in vivo* of gene marked cells derived from a long-lived CD34<sup>+</sup> progenitor cell transduced in the presence of CH-296 was almost one log higher than that of those transduced by co-culture<sup>20</sup>. In a preliminary report<sup>36</sup>, two patients with severe combined immunodeficiency were treated on a gene therapy trial using CH-296 and a retroviral vector expressing the common gamma chain of interleukin 2 receptor. There was substantial correction of the disease phenotype in lymphoid elements of both patients<sup>36</sup>. Our data, in addition to many other studies (including those in nonhuman primates), indicate CH-296 may be involved in the increased gene transfer reported here.

The optimum cytokine combination for increasing gene transfer into human stem and progenitor cells remains to be defined. SCF/MGDF/G-CSF has been shown to yield a reproducibly higher level of gene transduction of progenitor cells compared with that of CSF/IL-6 (ref. 32). Accordingly, we amended our protocol, and the CD34<sup>+</sup> cells from the last two patients were transduced using SCF/MGDF/G-CSF. These two patients had the highest gene marking of bone marrow colonies

one month after transplant and the highest levels of transduced cells in the peripheral blood, and these cells persisted throughout the observation period (9 months). Unfortunately, the two patients relapsed approximately 7 months after transplantation, preventing longer follow-up. Alternate cytokine combinations may provide further improvements. The addition of FLT-3 ligand to IL-3/IL-6/SCF improves gene transfer in nonhuman primates<sup>37</sup>. There was similar long-term marking of peripheral blood cells in a canine study using G-CSF/SCF/FLT-3 ligand and CH-296 (ref. 38).

Our data demonstrate that exposure of CD34<sup>+</sup> cells to CH-296 did not adversely affect engraftment kinetics. Hematopoietic function has remained normal in the eight surviving patients with almost 2 years of follow-up. This is an encouraging finding, given a recent report on the development of myeloproliferative syndrome in mice transplanted with MDR-1-transduced marrow cells<sup>39</sup>. At present, we do not know whether expression of MDR-1 or other factors led to the hematologic disorder<sup>39</sup>. However, our study differs in several ways from that mouse study<sup>39</sup>. A mutated version of the MDR-1 cDNA that contains a substitution of valine with glycine at position 185 was used in the mouse study, and the transduced cells were expanded *in vitro* for a much longer time (up to 12 days)<sup>39</sup>. Multiple integrations may have occurred as a result of this transduction procedure, increasing the risk of insertional mutagenesis<sup>31</sup>. In contrast to that study, abnormal hematopoiesis was not reported in four human studies using MDR-1 or in many other mouse transplant experiments using the A12M1 vector<sup>5,6,29,30,40</sup>. As prior human gene therapy studies of MDR-1 attained only lower levels of transduction and our study showed that most transduced cells contained a truncated MDR-1 gene, the propensity of MDR-1 vectors to elicit human myeloproliferative disorders remains to be determined.

In conclusion, we have demonstrated safe and efficient retrovirus-mediated gene transfer into cytokine-stimulated, CD34<sup>+</sup> hematopoietic cells transduced using CH-296. Our findings are relevant to most gene therapy approaches targeting hematopoietic cells and indicate therapeutic levels of gene-transduced cells can be attained for a variety of genetic and malignant diseases.

## Methods

**Patient selection.** Patients with documented germ cell tumors who had refractory or relapsed disease enrolled on this study. Eligibility criteria included an Eastern Cooperative Oncology Group performance status of 2 or less, normal cardiac function, normal liver function tests, creatinine clearance of more than 60 ml/min, diffusion capacity of more than 50%, and absence of active infections. All patients met the pre-transplant assessment criteria according to our institutional protocol and signed an informed consent form conforming to our institutional review board guidelines.

**Mobilization and isolation of CD34 cells.** PBPCs were mobilized using 10 µg G-CSF/kg per day (Amgen, Thousand Oaks, California) subcutaneously for 4 d. Leukapheresis was initiated using the mononuclear cell collection procedure of the COBE Spectra Cell Separator (COBE Laboratories, Lakewood, Colorado). Leukapheresis goals were a minimum of  $2 \times 10^6$  CD34<sup>+</sup> cells/kg for gene transfer and  $1 \times 10^9$  mononuclear cells/kg for the first transplantation and for 'back-up' to be used in case of graft failure of the transduced cells. A maximum of 5 d of leukapheresis were allowed. The Isolux® 300i system (Nexell Therapeutics, Irvine, California) was used to process PBPCs and select CD34<sup>+</sup> cells according to the manufacturer's recommendations.

**Retroviral vector and transduction protocol.** Genetix Pharmaceuticals (Cambridge, Massachusetts) provided the AM12M1 vector. This vector con-

tains the MDR-1 cDNA within the Harvey murine sarcoma virus long terminal repeats and was packaged by the AM12 amphotrophic packaging cell line. The protocol has received approval from the National Institutes of Health Recombinant DNA Advisory Committee and the Food and Drug Administration, and the vector used in this trial was produced at the National Gene Vector Laboratory (Indianapolis, Indiana). Cells were cultured in Iscove's Modified Dulbecco's Media (IMEM, BioWhittaker, Walkersville, Maryland) with 10% fetal calf serum (Hyclone, Logan, Utah) containing SCF/IL-6 (R&D Systems, Minneapolis, Minnesota) or G-CSF/MGDF/SCF (at final concentration of 100 ng/ml each) (Amgen, Thousand Oaks, California). The final cell concentration was  $5 \times 10^5$ – $1 \times 10^6$  cells/ml. After 48 h of prestimulation,  $4 \times 10^7$  CD34<sup>+</sup> cells were plated on non-tissue culture dishes treated with the fibronectin fragment CH-296 (Takara Shuzo, Otsu, Japan). Cells were exposed to vector for 4 h, then collected and resuspended in fresh media with cytokines; the next day, the transduction was repeated and the cells were cultured for an additional 8–16 h before being cryopreserved. A portion of the transduced cells were removed before the cryopreservation to document vector integration, expression of MDR-1 and the absence of replication-competent retrovirus.

**Transplantation regimen.** Patients received etoposide (750 mg/m<sup>2</sup> per day, intravenously over 2 h) and carboplatin (700 mg/m<sup>2</sup> per day, intravenously over 30 min) on days 6, 5 and 4 before the infusion of stem cells (Fig. 1). Patients also received prophylactic quinolone antibiotics and flucanazole. Frozen cell products were brought to the patient's room, thawed, and infused without delay. All patients received 5 µg G-CSF/kg per day until absolute neutrophil counts were  $2 \times 10^9$  cells/l for 2 consecutive days. After hematologic recovery from cycle 2 ( $3 \times 10^9$  or more white blood cells/l; absolute neutrophil counts,  $1.5 \times 10^9$  or more cells/l; and  $75 \times 10^9$  or more platelets/l), Etoposide was administered orally at a dose of 50 mg twice a day for 21 consecutive days every 4 weeks for three cycles. If absolute neutrophil counts decreased to less than  $0.5 \times 10^9$  cells/l, then etoposide was stopped and the doses of the following cycles were reduced by 25%. Subsequent cycles were started when absolute neutrophil counts were more than  $1.5 \times 10^9$  cells/l and platelet counts were more than  $75 \times 10^9$  platelets/l.

**Hematopoietic progenitor assays and drug resistance.** PBSCs or bone marrow cells ( $5 \times 10^2$  for CD34-selected and  $5 \times 10^4$  for marrow cells) were 'seeded' in plastic 35-mm tissue culture dishes containing 1.1% methylcellulose (Stem Cell Technologies, Vancouver, Canada). Media contained 1 ml 30% FBS, 50 µM β-mercaptoethanol, cytokines (50 ng/ml SCF; 10 ng/ml GM-CSF, 10 ng/ml IL-3 and 3 U/ml erythropoietin). Cultures were incubated at 37 °C in an atmosphere of 100% humidity and 5% CO<sub>2</sub>. Erythroid burst-forming units, granulocyte-monocyte CFU, and granulocyte, erythroid, macrophage and megakaryocyte CFU were counted *in situ* after 14 d. Paclitaxel was used at a dose of 10 ng/ml for the drug-resistance assay (Bristol-Myers Squibb, Princeton, New Jersey). Total cellular DNA was isolated from individual colonies on day 14 for PCR analysis. Colonies were assigned scores, and then DNA was isolated for PCR. Each colony was placed into 150 µl PBS (Sigma), 150 µl phenol-chloroform-isoamyl alcohol (Roche Diagnostics, Indianapolis, Indiana) was added, and each tube was vortexed well. Samples were incubated on ice for 15 min, and then centrifuged at 12,000g for 10 min at 4 °C. The upper aqueous phase was transferred to a clean tube containing 500 µl isopropanol (Sigma). DNA was precipitated overnight at –20 °C, and then samples were centrifuged at 12,000g at 4 °C for 20 min to pellet DNA; the pellets were washed with 70% ethanol. Pellets were air-dried and then resuspended in 30 µl of water. Care was taken to exclude adjacent colonies to prevent cross-contamination and overestimation of gene transfer efficiency by nested PCR. To confirm the validity of this method, we mixed MDR-1-transduced CD34<sup>+</sup> cells and cytokine-stimulated control CD34<sup>+</sup> cells at various ratios. These cells were then plated in methylcellulose, and the gene transfer efficiency was calculated for each ratio. As the percent of MDR-1-transduced CD34<sup>+</sup> cells decreased, there was a corresponding decrease in the number of transgene-containing colonies. Many colonies were screened per patient, and only those with a sufficient amount of recovered DNA, as judged by the presence of β-globin DNA, were included in the calculation of gene transfer efficiency.

**Nested and quantitative PCR.** PCR reactions used 10 µl DNA in a total volume of 50 µl per reaction containing 2 U AmpliTaq DNA polymerase (Perkin

Elmer, Foster City, California). Both rounds of MDR-1 PCR consisted of 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. Primers for the first round were 5'-GCCACATCATCATGATC-3' and 5'-GTCTCTACTT-TAGTGCT-3' (494-base-pair product)<sup>31</sup>. The second round used primers 5'-ACGGAAGGCCTAATGCCG-3' and 5'-TGACTCGATGAAGGCATG-3' (414-base-pair product). As a control for the presence of colony DNA, PCR was used to assess the presence of the β-globin gene with the primers 5'-GAATCCAGATGCTCAAGGCC-3' and 5'-CAATCCAGCTACCATTCTGC-3' (344-base-pair product). For nested PCR,  $1 \times 10^6$ – $5 \times 10^6$  bone marrow or peripheral blood cells were lysed using 0.6 ml Cell Lysis Solution (Puregen; Gentra Systems, Minneapolis, Minnesota). For quantitative PCR, TagMan Multiplex PCR (PE Applied Biosystems, Foster City, California) for both MDR1-cDNA and ApoB gene was used. DNA (0.5 µg) was plated in a 96-well optical tray with optical caps (MicroAmp; Perkin Elmer, Norwalk, Connecticut). The final reaction mixture of 50 µl consisted of 1x TagMan buffer A (50 mM KCl, 10 mM Tris HCl, pH 8.3, 0.01 mM EDTA and 60 nM Passive Reference rhodamine carboxyl X), 5.5 mM MgCl<sub>2</sub>; 300 µM dATP, dCTP and dGTP, 600 µM dUTP; 300 nM 5' primer MDR1-F (5'-AG-GAAGCCAATGCTATGACTTTA-3'); 300 nM 3' primer MDR1-R (5'-AATG-CGCATCCTCTGCTCTG-3'); 200 nM fluorescent probe TP-MDR1 (5'-carboxyfluorescein-CATGAACTGCCTCATAAATTTGACCCCTG-N,N,N',N'-tetramethyl-6-carboxyrhodamine-3'); 80 nM 5' primer ApoB-F (5'-TGAAGCGTGGAGGACATTCCTCTA-3'); 80 nM 3' primer ApoB-R (5'-CTGGAATTGCGATTCTGCTGTA-3'); 200 nM fluorescent probe TP-ApoB (5'-VIC, -CGAGAATCACCCCTGCCAGATCTCCGT-N,N,N',N'-tetramethyl-6-carboxyrhodamine-3'); 0.5 U AmpErase UNG (uracil-N-glycosylase); and 1.25 U AmpliTaq Gold DNA Polymerase (Perkin Elmer, Norwalk, Connecticut). The set of primers and probes that detect both full-length and truncated transgene were MDR-F3 (5'-GAAGAAGGCCAGACCCGCTG-3'), MDR-R3 (5'-ACAGGATGGGCTCCTGGG-3') and TP-MDR3 (5'-carboxyfluorescein-CAGTGGCTCCGAGCACACCTGG-N,N,N',N'-tetramethyl-6-carboxyrhodamine-3'). All samples were assayed in triplicate. The standard curves were of hematopoietic cell lines (KG1 and U937) transduced with A12M1 and MDR-1. PCR amplification used an ABI PRISM™ Sequence Detection System (PE Applied Biosystems, Foster City, California) and the following parameters: 50 °C for 2 min (UNG incubation), 95 °C for 10 min to activate the Taq DNA polymerase, then 50 cycles of 95 °C for 15 s (denaturation) followed by 60 °C for 1 min (annealing and extension).

**Safety assessment.** The safety of the retrovirus-mediated gene transfer was assessed by analysis for the presence of both replication-competent retrovirus and viral envelope. Mus dunni cells ( $1 \times 10^6$ ) were co-cultured for 3 weeks, then tested by the sarcoma-positive/leukemia-negative (S<sup>+</sup>/L<sup>-</sup>) assay<sup>42</sup>. Results were available before the infusion of transduced cells. PCR for viral envelope was done on all transduced products and after transplantation samples as described<sup>43</sup>.

Antibodies against CH-296 were tested by analysis of patient sera samples by immunoassay at an independent commercial facility (Tanox, Houston, Texas). Takara Shuzo (Otsu, Japan) developed both ELISA and western blot analyses for detecting antibodies against CH-296, and transferred the standard operating procedure to Tanox (Houston, Texas). Samples were considered positive if they exceeded ELISA limit and were confirmed by western blot analysis. Each patient serum sample was diluted 1:11. A mouse monoclonal antibody against CH-296 was used as the positive control. Samples from normal human serum (WAKO) were used as the negative control. Samples with ratio exceeding the mean + 3 s.d. of the negative control on two separate analyses were assigned scores as potential positives and were subjected to confirmatory western blot analysis. For western blot analyses, CH-296 was reduced and denatured, then separated by standard SDS-PAGE and blotted into a PVDE membrane. This membrane was blocked with horse serum and divided into three pieces. One was incubated with the mouse antibody against CH-296; a second, with WAKO at 1:11 dilution; and a third, with the sample at a dilution of 1:11. The expected band size for CH-296 was 63 kDa.

#### Acknowledgments

We thank the patients and their families for their contributions to this study. We thank the Indiana University General Clinical Research Center, Stem Cell Laboratory (V. Graves and J. Bally), Apheresis, and BMT unit staff for

## ARTICLES

excellent technical and clinical care. We thank S. Harker from the General Clinical Research Center nursing staff for her contribution to this study. We thank the members of the Gene Therapy Working Group for their contribution (L. Rubin, J. Croop, M. Dinuer, S. Richeson, L. Reeves, J. Good and S. Holbrook). This work was supported by the National Centers for Research Resources (National Institutes of Health M01 RR00750) (R.A.) and an American Cancer Society grant CRTG-97-042-EDT (R.A.). The National Gene Vector Laboratory (U42 RR/1148 and CA11148) produced the clinical-grade vector. Additional support was provided by the Indiana University Vector Core and the Stem Cell Laboratory Core (2 P30 DK49218 and P01 HL 53586) (K.C. and R.A.). Takara Shuzo (Otsu, Japan) has provided the Gene Therapy Working Group with an unrestricted grant to help conduct clinical gene therapy trials at our center. A.B. has an equity interest in Genetix Pharmaceuticals, which provided the vector-packaging cell line (A12M1) to the National Gene Vector Laboratory for this study. D.A.W. may receive royalties based on 'milestones' set forth in a licensing agreement between Takara Shuzo and Indiana University.

RECEIVED 17 FEBRUARY; ACCEPTED 18 APRIL 2000

- Kohn, D.B. *et al.* A clinical trial of retroviral-mediated transfer of a rev-responsive element decay gene into CD34(+) cells from the bone marrow of human immunodeficiency virus-1-infected children. *Blood* **94**, 368–371 (1999).
- Kohn, D.B. *et al.* T lymphocytes with a normal ADA gene accumulate after transplantation of transduced autologous umbilical cord blood CD34+ cells in ADA-deficient SCID neonates. *Nature Med.* **4**, 775–780 (1998).
- Dunbar, C. & Kohn, D. Retroviral mediated transfer of the cDNA for human glucocerebrosidase into hematopoietic stem cells of patients with Gaucher disease. A phase I study. *Hum. Gene Ther.* **7**, 231–253 (1996).
- Brenner, M.K. *et al.* Gene marking to determine whether autologous marrow infusion restores long-term haemopoiesis in cancer patients. *Lancet* **342**, 1134–1137 (1993).
- Hesdorffer, C. *et al.* Phase I trial of retroviral-mediated transfer of the human MDR1 gene as marrow chemoprotection in patients undergoing high-dose chemotherapy and autologous stem-cell transplantation. *J. Clin. Oncol.* **16**, 165–172 (1998).
- Hanania, E.G. *et al.* Results of MDR-1 vector modification trial indicate that granulocyte/macrophage colony-forming unit cells do not contribute to posttransplant hematopoietic recovery following intensive systemic therapy. *Proc. Natl. Acad. Sci. USA* **93**, 15346–15351 (1996).
- Kohn, D. B. *et al.* Engraftment of gene-modified umbilical cord blood cells in neonates with adenosine deaminase deficiency. *Nature Med.* **1**, 1017–1023 (1995).
- Dunbar, C.E. *et al.* Retrovirally marked CD34-enriched peripheral blood and bone marrow cells contribute to long-term engraftment after autologous transplantation. *Blood* **85**, 3048–3057 (1995).
- Emmons, R.V.B. Retroviral gene transduction of adult peripheral blood or marrow-derived CD34+ cells for six hours without growth factors or on autologous stroma does not improve marking efficiency assessed *in vivo*. *Blood* **89**, 4040–4046 (1997).
- Orlic, D. *et al.* Identification of human and mouse hematopoietic stem cell populations expressing high levels of mRNA encoding retrovirus receptors. *Blood* **91**, 3247–3254 (1998).
- Dunbar, C.E. *et al.* Improved retroviral gene transfer into murine and Rhesus peripheral blood or bone marrow repopulating cells primed *in vivo* with stem cell factor and granulocyte colony-stimulating factor. *Proc. Natl. Acad. Sci. USA* **93**, 11871–11876 (1996).
- MacNeill, E.C. *et al.* Simultaneous infection with retroviruses pseudotyped with different envelope proteins bypasses viral receptor interference associated with colocalization of gp70 and target cells on fibronectin CH-296. *J. Virol.* **73**, 3960–3967 (1999).
- Traycoff, C.M. *et al.* The 30/35 kDa chymotryptic fragment of fibronectin enhances retroviral-mediated gene transfer in purified chronic myelogenous leukemia bone marrow progenitors. *Leukemia* **11**, 159–167 (1997).
- Moore, K.A. *et al.* Stromal support enhances cell-free retroviral vector transduction of human bone marrow long-term culture-initiating cells. *Blood* **79**, 1393–1399 (1992).
- Moritz, T., Patel, V.P. & Williams, D.A. Bone marrow extracellular matrix molecules improve gene transfer into human hematopoietic cells via retroviral vectors. *J. Clin. Invest.* **93**, 1451–1457 (1994).
- Baum, C. *et al.* Novel retroviral vectors for efficient expression of the multidrug resistance (mdr-1) gene in early hematopoietic cells. *J. Virol.* **69**, 7541–7547 (1995).
- Thomsen, S. *et al.* Lack of functional Pit-1 and Pit-2 expression on hematopoietic stem cell lines. *Acta Haematol.* **99**, 148–155 (1998).
- Kotani, H. *et al.* Improved methods of retroviral transduction and production for gene therapy. *Hum. Gene Ther.* **5**, 19–28 (1994).
- Hanenberg, H. *et al.* Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nature Med.* **2**, 876–882 (1996).
- Kiem, H.P. *et al.* Improved gene transfer into baboon marrow repopulating cells using recombinant human fibronectin fragment CH-296 in combination with interleukin-6, stem cell factor, FLT-3 ligand, and megakaryocyte growth and development factor. *Blood* **92**, 1878–1886 (1998).
- Cooper, M.A. & Einhorn, L.H. Maintenance chemotherapy with daily oral VP-16 following salvage therapy in patients with germ cell tumors. *J. Clin. Oncol.* **13**, 1167–1169 (1995).
- Ueda, K. *et al.* Expression of a full-length cDNA for the human 'MDR1' gene confers resistance to colchicine, doxorubicin and vinblastine. *Proc. Natl. Acad. Sci. USA* **84**, 3004–3008 (1987).
- Ward, M. *et al.* Transfer and expression of the human multiple drug resistance gene in human CD34+ cells. *Blood* **84**, 1408–1414 (1994).
- Fan, Y. *et al.* Detection of germ cell tumor cells in apheresis products using polymerase chain reaction. *Clin. Cancer Res.* **4**, 93–98 (1998).
- Chou, P.M. *et al.* Differential expression of p53, c-kit, and CD34 in prepubertal and postpubertal testicular germ cell tumors. *Cancer* **79**, 2430–2434 (1997).
- Galipeau, J. *et al.* A bicistronic retroviral vector for protecting hematopoietic cells against antifolates and P-glycoprotein effluxed drugs. *Hum. Gene Ther.* **8**, 1773–1783 (1997).
- Sorrentino, B.P. *et al.* Expression of retroviral vectors containing the human multidrug resistance 1 cDNA in hematopoietic cells of transplanted mice. *Blood* **86**, 491–501 (1995).
- Ward, M. *et al.* Retroviral transfer and expression of the human multiple drug resistance (MDR) gene in peripheral blood progenitor cells. *Clin. Cancer Res.* **2**, 873–876 (1996).
- Moscow, J.A. *et al.* Engraftment of MDR1 and NeoR gene-transduced hematopoietic cells after breast cancer chemotherapy. *Blood* **94**, 52–61 (1999).
- Cowan, K.H. *et al.* Paclitaxel chemotherapy after autologous stem-cell transplantation and engraftment of hematopoietic cells transduced with a retrovirus containing the multidrug resistance complementary DNA (MDR1) in metastatic breast cancer patients. *Clin. Cancer Res.* **5**, 1619–1628 (1999).
- Cornetta, K. Safety aspects of human gene therapy. *Br. J. Haematol.* **80**, 421–426 (1992).
- Hanenberg, H. *et al.* Optimization of fibronectin-assisted retroviral gene transfer into human CD34+ hematopoietic cells. *Hum. Gene Ther.* **82**, 2193–2206 (1997).
- Hennemann, B. *et al.* Optimization of retroviral-mediated gene transfer to human NOD/SCID mouse repopulating cord blood cells through a systematic analysis of protocol variables. *Exp. Hematol.* **27**, 817–825 (1999).
- Stewart, A.K. *et al.* Engraftment of gene-marked hematopoietic progenitors in myeloma patients after transplant of autologous long-term marrow cultures. *Hum. Gene Ther.* **10**, 1953–1964 (1999).
- Dao, M.A. *et al.* Adhesion to fibronectin maintains regenerative capacity during ex vivo culture and transduction of human hematopoietic stem and progenitor cells. *Blood* **92**, 4612–4621 (1998).
- Cavazzana-Calvo, M. *et al.* Correction of SCID-X1 disease phenotype following  $\gamma$  c gene transfer by a retroviral vector into CD34+ cells in two children. *Blood* (suppl. 1) **94**, 367a (1999).
- Tisdale, J.F. *et al.* Ex vivo expansion of genetically marked rhesus peripheral blood progenitor cells results in diminished long-term repopulating ability. *Blood* **92**, 1131–1141 (1998).
- Goerner, M. *et al.* The use of granulocyte colony-stimulating factor during retroviral transduction on fibronectin fragment CH-296 enhances gene transfer into hematopoietic repopulating cells in dogs. *Blood* **94**, 2287–2292 (1999).
- Bunting, K.D. *et al.* Transduction of murine bone marrow cells with an MDR1 vector enables ex vivo stem cell expansion, but these expanded grafts cause a myeloproliferative syndrome in transplanted mice. *Blood* **92**, 2269–2279 (1998).
- Richardson, C. & Bank, A. Preselection of transduced murine hematopoietic stem cells populations leads to increased long-term stability and expression of human multiple drug resistance gene. *Blood* **86**, 2579–2589 (1995).
- Sorrentino, B. *et al.* Selection of drug-resistant bone marrow cells in vivo after retroviral transfer of human MDR1. *Science* **257**, 99–103 (1992).
- Cometta, K. *et al.* Infection of human cells with murine amphotropic replication-competent retroviruses. *Hum. Gene Ther.* **4**, 579–588 (1993).
- Morgan, R.A., Cornetta, K. & Anderson, W.F. Application of polymerase chain reaction in retroviral-mediated gene transfer and the analysis of gene-marked human TIL cells. *Hum. Gene Ther.* **1**, 136–149 (1990).